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PATENT SPECIFICATION

NO DRAWINGS

1,166,896

1,166,896

Date of Application (No. 16727/67) and filing Complete Specification: 12 April, 1967.

Application made in Japan (No. 22942) on 13 April, 1966.

Complete Specification Published: 15 October, 1969.

Index at Acceptance:—C2 C3A18.

International Classification:—C 13 k 9/00.

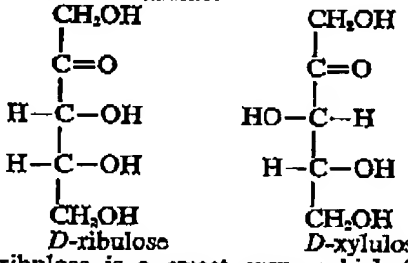
COMPLETE SPECIFICATION

Process for producing D-Ribulose and D-Xylulose by fermentation

We, KYOWA HAKKO KOGYO CO., LTD., a corporation organised under the laws of Japan, of 4, Ohtemachi-1-chome, Chiyoda-ku, Tokyo, Japan, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the followng statement:—

The present invention relates to a method for producing *D*-ribulose and/or *D*-xylulose by fermentation. More particularly, the present invention relates to a process for producing *D*-ribulose and *D*-xylulose by culturing microorganisms belonging to the genus *Brevibacterium* or the genus *Corynebacterium* in an aqueous nutrient medium containing at least one source of carbon, under aerobic conditions.

D-ribulose and *D*-xylulose are ketopentoses having, respectively, the following structural formulae:



D-ribulose

D-xylulose

D-ribulose is a sweet syrup which finds use as a food addition and medicine. *D*-xylulose is useful as a food addition and medicine. Hence, it would be quite advantageous to have available an effective process for producing the same.

Accordingly, one of the objects of the present invention is to provide a process for the production of *D*-ribulose and/or *D*-xylulose.

Another object of the present invention is to provide a process for producing *D*-ribulose and *D*-xylulose by fermentation which may be carried out in an efficacious and simple manner.

A further object of the invention is to provide a process for producing *D*-ribulose and *D*-xylulose by fermentation which may be carried out advantageously on an industrial scale at a reasonable cost to give a high yield of product.

These and other objects and advantages of the present invention will become apparent to those skilled in the art from a consideration of the following specification and claims.

In accordance with the present invention, it has been found that certain species of microorganisms which are widely present in nature are capable of forming and accumulating large amounts of *D*-ribulose and/or *D*-xylulose in an aqueous nutrient culture medium. These microorganisms have been identified by the present inventors as belonging to the genus *Brevibacterium* and the genus *Corynebacterium*.

Particular strains within these genera, as discussed below, exhibited bacteriological properties which were not in accordance with those of well-known bacteria. Accordingly, it was acknowledged that these strains were novel strains. The identification thereof was carried out with reference to the "Manual of Microbiological Methods" (Society of American Bacteriologists).

These particular strains were isolated from natural sources and exhibit the properties shown hereinbelow.

[Price 4s. 6d.]

Table 1

A. Observation of Properties Upon Culturing:

	12-6 strain	22-23 strain
Microscopic observation	Rod-shaped cells, 0.6-0.8×1.5 microns, rarely rod shaped, 0.6-0.8×3-4 microns occasionally appear, no branching and rounded ends, some are in clubbed shape, no V-shaped cells	Rods or short rods, 0.8×1.2-1.5 microns; rarely stretched, branching according to conditions, rounded ends
Gram-stain	positive	positive
Agar plate at 30°C. for 2 days (Bouillon Agar)		
Size	1.5-2 mm (diameter)	1.5-2 mm (diameter)
Shape	round	round
Surface	flat and smooth	flat and smooth
Rising	half-lens form	half-lens form
Periphery	complete periphery	complete periphery
Content	homogeneous	homogeneous
Color	cream	creamy-yellow
Transparency	opaque	opaque
Luster	wet light	wet light
Quality	butyrous	butyrous
Slant culture at 30°C. for 2 days (Bouillon Agar)		
Growth	good	good
Form of colony	wide form	wide form
Rising of the section	stand-form	stand-form
Color	cream	creamy-yellow
Stub culture at 30°C. for 2 days (Bouillon Agar)		
Growing position	The upper portion is better than the lower.	The upper portion is better than the lower.
Form of colony	thread-form-chain-form	thread-form-chain-form
Liquid culture	no generation of gas	no generation of gas
Growth of the surface	cell ring	cell ring
Turbidity	slightly hazy	slightly hazy
Precipitation	viscous	viscous
Gas	not generated	not generated
Coloring	none	none
Liquefaction of gelatin	none	none
Growth on potato	thread-form	thread-form

B. Observation of Physiological Properties:

	12-6 strain	22-23 strain
Reduction of nitrate	positive (a little weak)	positive
Formation of indole	negative	negative
Formation of hydrogen sulfide	positive	positive
Hydrolysis of starch	negative	negative
Formation of acetylmethylcarbinol	negative	negative
Litmus milk	reduced	reduced
B C P milk	unchanged	unchanged
Formation of acid from saccharides	No formation of acid from 20 kinds of saccharides, no formation of gas	No formation of acid from 20 kinds of saccharides, no formation of gas
Optimum temperature for growth	30°C.	30°C.

The above results were examined with reference to "Bergey's Manual of Determinative Bacteriology", 7th Edition (1957). It is considered that the 12-6 strain belongs to the genus *Brevibacterium* because it has no branching in the shape thereof and is clearly gram-positive. Moreover, several other bacteriological properties are similar to those found for *Brevibacterium* species. On the other hand, the 22-23 strain sometimes shows a branched structure, which is different from the 12-6 strain. It is considered from this and several other bacteriological properties that the 22-23 strain is one of the *Corynebacteriaceae*. The 22-23 strain grows at 37°C. and shows the properties of being gram-positive and not liquefying gelatin, so that it should belong to the genus *Corynebacterium*.

The 12-6 strain is different from *Brevibacterium vitarumen* with respect to the utilization of saccharides, different from *Brevibacterium maris* with respect to the utilization of cassharides, its optimum temperature for growth and its behaviour with respect to litmus milk, and also different from *Brevibacterium ammoniagenes* in regard to its behaviour as to litmus milk, its degree of utilization of saccharides and the color of its colony. Accordingly, there is no known species corresponding to the 12-6 strain.

The 22-23 strain is similar to *Corynebacterium pseudodiphtheriticum*, but slightly different therefrom with respect to the color of its colony. Additional information in the above-noted Bergey's Manual is insufficient to make further comparisons.

From the above, it was concluded that both the 12-6 strain and the 22-23 strain are novel strains of *Brevibacterium* and *Corynebacterium*, respectively. These have been named, respectively, *Brevibacterium NOV. SP.*, and *Corynebacterium NOV. SP.* and have been deposited with the American Type Culture Collection, whereby they have been given ATCC No. 21049 and ATCC No. 21050, respectively.

The detection of the saccharides accumulated in the fermentation liquor as a result of culturing the above strains can be carried out by paper chromatography employing various developing agents. Thus, the desired saccharide derivatives can be isolated e.g. by an ion exchange treatment and then identified by the procedure described hereinbelow.

After culturing has been carried out for some time, the cells are removed by centrifugal separation, and the supernatant liquid is concentrated under reduced pressure. Thereafter, 95% ethanol is added to remove the precipitated polysaccharides. After further concentration, the resulting solution is dissolved in 1/20 M potassium tetraborate. The resultant solution is then poured into a column filled with a strongly basic anion exchange resin (Dowex-1, borate type) and

is eluted with a 0.02 M-0.04 M potassium tetraborate solution, thereby obtaining a ribulose fraction and a xylulose fraction. Each fraction is treated with Dowex-50 (H^+ type) to remove potassium and then concentrated. ("Dowex" is a registered Trade Mark).

Thereafter, anhydrous methanol is added in order to remove the methyl borate. Decoloring is carried out by adding active carbon. In the case of ribulose, it is separated as the ortho-nitrophenylhydrazone. Xylulose is separated as the para-bromophenylhydrazone. These products are then re-crystallized with anhydrous alcohol (for example, methanol or ethanol).

The ortho-nitrophenylhydrazone derivative has a melting point of 165°C., $[\alpha]_D^{20} = -46.5°$, and the para-bromophenylhydrazone derivative has a melting point of 126°C., $[\alpha]_D^{20} = +25.0°$ (after 15 minutes), and $[\alpha]_D^{20} = -32.5°$ (after 1 week). These values accord completely with standard reference materials of ribulose ortho-nitrophenylhydrazone and xylulose para-bromophenylhydrazone, respectively. Moreover, the free saccharide products prepared by hydrolyzing the above-mentioned saccharide derivatives and developing the same by the cysteinecarbazole sulfuric acid method, or by reacting them with orcinol reagent, give extinction curves for the developed solutions which accord completely with those of the standard materials, respectively. Furthermore, the R_f value obtained by paper chromatography with a phenol-water system and other solvents shows that the obtained saccharides are ribulose and xylulose, respectively.

Accordingly, it is clear that the products produced by the above-described strains in a fermentation method are *D*-ribulose and *D*-xylulose.

The fermentation employed for obtaining the products of the present invention may be carried out either in a synthetic culture medium or a natural nutrient medium as long as it contains the essential nutrients for the growth of the strain employed. Such nutrients are well known in the art and include substances such as a carbon source, a nitrogen source and inorganic compounds which are utilized by the microorganism employed in appropriate amounts. Thus, as a carbon source, there may be mentioned, by way of example, an appropriate amount of at least one mono- or poly-saccharide, such as glucose, fructose, maltose, sucrose, starch, starch hydrolysate or molasses, or any other suitable carbon source such as glycerol, mannitol, sorbitol; or organic acids. These substances may be used either singly or in mixtures of two or more. As a nitrogen source, various kinds of inorganic or organic salts or compounds, such as urea or ammonium salts such as ammonium chloride, ammonium sulfate, ammonium nitrate or ammonium phosphate or natural substances containing nitrogen, such as cornsteep liquor, yeast extract, meat extract or peptone, fish meal, bouillon, casein hydrolysates, fish solubles or rice bran extract, may be employed. Again, these substances may also be used either singly or in combinations of two or more. Inorganic compounds which may be added to the culture medium include magnesium sulfate, sodium phosphate, potassium dihydrogen phosphate, potassium monohydrogen phosphate, iron sulfate or other iron salts, manganese chloride or calcium chloride.

Culturing is carried out under aerobic conditions, such as aerobic shaking of the culture or with stirring of a submerged culture at a temperature of 20° to 40°C. and at a pH of 5.0 to 9.0. After about 2 to 8 days of culturing under these conditions, remarkably large amounts of *D*-ribulose and/or *D*-xylulose are found to be accumulated in the culture medium. These ketopentoses may then be recovered in a suitable manner, such as the ion exchange resin treatment described hereinabove.

The following examples are illustrative of the present invention. Unless otherwise noted, the percentages therein are by weight.

Example 1

Brevibacterium NOV. SP. No. 12-6 ATCC 21049 is employed as the seed strain. Culturing is carried out therewith with aerobic shaking for 24 hours in a seed culture medium comprising 2% of glucose, 1% of yeast extract, 1% of peptone and 0.25% of sodium chloride in a conical flask. Thereafter, 2 ml. of the seed culture medium is transferred into a 250 ml. conical flask containing 20 ml. of the following fermentation medium.

10.0% glucose
1.0% potassium monophosphate
1.0% potassium diphosphate
1.0% magnesium sulfate heptahydrate
0.6% urea
0.5% yeast extract
0.01% calcium chloride dihydrate
30 γ/l biotin

The pH of the fermentation medium before sterilization is 8.0. As is conventional, the medium is contained in distilled water.

Fermentation is then carried out with aerobic shaking at 30°C. for 4-5 days. After 5 days of culturing, 10.3 mg/ml. of *D*-ribulose and 11.4 mg/ml. of *D*-xylulose are found to be accumulated in the culture medium. These products may then be separated therefrom as desired, for example, by an ion exchange resin treatment and the necessary subsequent steps.

Example 2

Culturing is carried out in the same manner and in the same medium as described in Example 1 except that *Corynebacterium NOV. SP.* No. 22-23 ATCC 21050 is employed as the seed microorganism. As a result, 15.2 mg/ml. of *D*-ribulose and 12.8 mg/ml. of *D*-xylulose are accumulated in the culture medium.

WHAT WE CLAIM IS:—

1. A process for producing *D*-ribulose and *D*-xylulose which comprises culturing a microorganism *Brevibacterium* NOV. SP. No. 12-6 ATCC 21049 or *Coryneabacterium* NOV. SP. No. 22-23 ATCC 21050, in an aqueous nutrient medium containing at least one source of carbon under aerobic conditions.

2. The process of claim 1, wherein the culturing is carried out at a temperature of between 20° and 40°C and a pH of 5.0 to 9.0.

3. The process of claim 1, wherein the obtained *D*-ribulose and *D*-xylulose are recovered from the fermentation liquor by an ion exchange resin treatment.

4. Process according to claim 1 for producing *D*-ribulose of *D*-xylulose by fermentation substantially as described in either of the foregoing Examples.

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Printed for Her Majesty's Stationery Office by The Tweeddale Press Ltd., Berwick-upon-Tweed, 1969.
Published at the Patent Office, 25 Southampton Buildings, London, W.C.2, from which copies may be obtained.